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Replication-Independent Histone Deposition by the HIR Complex and Asf1

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Summary

The orderly deposition of histones onto DNA is mediated by conserved assembly complexes, including chromatin assembly factor-1 (CAF-1) and the Hir proteins [1–4]. CAF-1 and the Hir proteins operate in distinct but functionally overlapping histone deposition pathways in vivo [5, 6]. The Hir proteins and CAF-1 share a common partner, the highly conserved histone H3/H4 binding protein Asf1, which binds the middle subunit of CAF-1 as well as to Hir proteins [7–11]. Asf1 binds to newly synthesized histones H3/H4 [12], and this complex stimulates histone deposition by CAF-1 [7, 11, 12]. In yeast, Asf1 is required for the contribution of the Hir proteins to gene silencing [7, 13]. Here, we demonstrate that Hir1, Hir2, Hir3, and Hpc2 comprise the HIR complex, which copurifies with the histone deposition protein Asf1. Together, the HIR complex and Asf1 deposit histones onto DNA in a replication-independent manner. Histone deposition by the HIR complex and Asf1 is impaired by a mutation in Asf1 that inhibits HIR binding. These data indicate that the HIR complex and Asf1 proteins function together as a conserved eukaryotic pathway for histone replacement throughout the cell cycle.

Results and Discussion

Asf1 and the Hir Proteins Exist as a Complex In Vivo

We first examined Asf1-containing protein complexes. A double affinity "TAP" tag containing the S peptide from RNase A [14] flanked by a TEV protease site and a minimal protein A binding domain (ZZ tag) [15, 16] was fused to the C terminus of Asf1 by genomic integration at the endogenous locus [17]. This *ASF1-STAP* allele was functional in vivo, because it did not cause sensitivity to hydroxyurea or cause growth or silencing defects in the absence of a CAF-1 subunit (data not shown). Asf1-STAP complexes were purified by using buffers containing 100 mM KCl [16]. After the second affinity step, the eluted protein complex was analyzed by SDS-PAGE and silver staining (Figure 1A, left), which revealed a predominant band at the approximate molecular weight of Asf1-STAP and multiple copurifying species. The eluted material was also directly analyzed by mass spectrometry (Figure 1B), which identified the checkpoint kinase Rad53, the four Hir proteins, and histones H3 and H4. The copurifying proteins were absent when the purification was performed at higher ionic strength (300 mM KCl; Figure S1A available in the Supplemental Data with this article online), suggesting that their recovery resulted from electrostatic interactions with Asf1 and not because of insolubility. Furthermore, ethidium bromide, which intercalates between DNA bases, did not disrupt any of the observed interactions (Figure S1B), suggesting that these protein-protein interactions are not bridged by DNA [18]. These data confirmed the known interactions between Asf1 and Rad53 [19, 20], and between Asf1 and histones H3/H4 [12]. This is the first evidence that Asf1 interacts with all four Hir proteins as a group.

These data suggested that the Hir proteins represent a macromolecular complex. To further examine Hir protein partners, we generated a *HIR2-STAP* allele. The *HIR2-STAP* allele was functional in vivo, because it did not cause synergistic growth or silencing defects in cells lacking a CAF-1 subunit (Figure S2). The Hir2-STAP purification was performed as described for Asf1-STAP, with the exception that the ionic strength was maintained at 300 mM KCl. In the final protein complex (Figure 1A, right), two predominant bands were observed at the predicted molecular weights of Hir2-STAP (101 kDa) and Hir3 (191 kDa). Mass spectrometry confirmed that Hir1, Hir3, and Hpc2 copurified with Hir2 (Figure 1B). We note that Hir1 (93 kDa) and Hir2-STAP (101 kDa) appear to comigrate and that Hpc2 does not stain well with silver. All other species identified were common contaminants of TAP tag purifications (data not shown). Moreover, Hir1, Hir3, and Hpc2 copurified with Hir2 in the presence of ethidium bromide (data not shown). These data confirmed that the four Hir proteins interact in vivo, forming what we term the HIR complex.

Asf1-HIR Complex Architecture

To further define the protein interaction relationships, Asf1-STAP-containing complexes were purified from yeast strains lacking individual *HIR* genes. In the absence of any one of the HIR complex members, the remaining Hir proteins were generally not detected in the mass spectrometric analysis of the Asf1-STAP complexes (Figure 1C). The one exception was in the Asf1-STAP complexes from a *hir3Δ* strain, in which a much reduced number of Hir2 peptides (three) was recovered. Western blot analyses of whole-cell extracts confirmed that protein levels of Asf1-STAP did not change in any of the *hir* mutants (data not shown). Likewise, steady-state levels of Hir2-STAP were unaltered in *hir* and *asf1* mutant cells (Figure S3A). We conclude that the interaction between Asf1 and the HIR complex is greatly reduced in the absence of any of the four Hir subunits. The minor recovery of Hir2 in *hir3Δ* cells suggests Hir3 may be a more peripheral subunit of the HIR complex.

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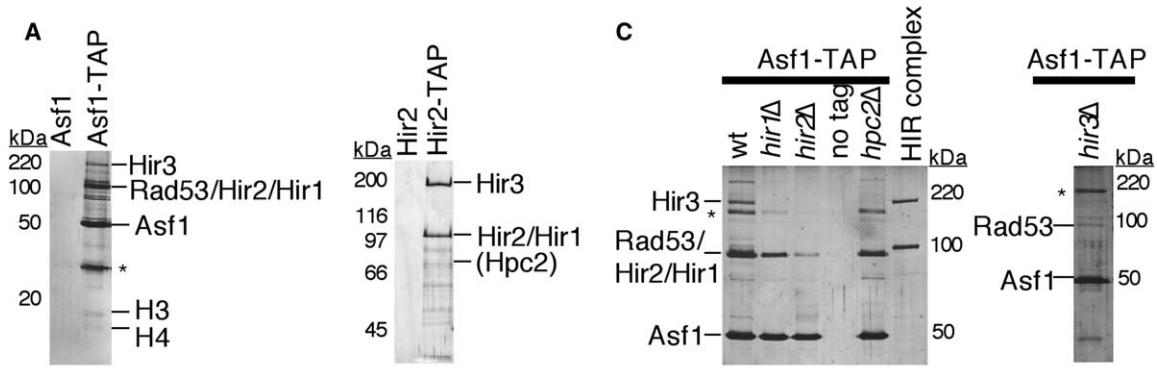

B

	Asf1-TAP	Hir2-TAP
Asf1	73.1(129)	-
Rad53	64.6(82)	-
Hir2	58.5(53)	54.9(69)
Hir1	49.5(44)	49.1(51)
Hir3	40.4(62)	45.0(85)
Hpc2	42.7(23)	76.1(122)
H4	49.5(6)	-

D

Protein	*hir1Δ*	*hir2Δ*	*hpc2Δ*	*hir3Δ*
Asf1	74.2(69)	73.1(54)	70.3(78)	56.6 (38)
Rad53	65.3(64)	51.6(34)	63.9(90)	36.9 (26)
H4	18.4(2)	-	18.4(3)	-
HIR	-	-	-	6.3(3)-Hir2 only

Figure 1. The Four Hir Proteins Exist as a Complex In Vivo and Copurify with Asf1

(A) TAP purifications were performed with wild-type (wt) (PKY028) and *ASF1-STAP* (PKY3121) cells (left) and from wt (PKY028) and *HIR2-STAP* cells (PKY3062, right). The Asf1-STAP purification was performed at 100 mM KCl, and the Hir2-STAP purification was performed at 300 mM KCl. Proteins eluted from the second TAP affinity resin were analyzed on an SDS-PAGE gel and detected by silver staining. Polypeptides identified by mass spectrometry, immunoblotting, or molecular weight (histone H3) are indicated. An asterisk (*) indicates an unidentified polypeptide.

(B) Summary of Asf1-STAP and Hir2-STAP mass spectrometry data. The percent sequence coverage is indicated in the table, with the number of unique peptides shown in parentheses. Although histone H3 peptides were not detected in all Asf1-STAP preparations by mass spectrometry, H3 was always apparent on the silver-stained protein gels.

(C) Efficient association between Asf1 and the HIR complex is dependent upon all four Hir proteins. Asf1-TAP complexes were purified from wt (PKY3121), *hir1Δ* (PKY3906), *hir2Δ* (PKY3908), *hpc2Δ* (PKY3912), and *hir3Δ* (PKY3928) cells, analyzed on SDS-PAGE gels, and detected by silver staining. Purified Hir2-STAP complex is shown for comparison. The Hir2-STAP subunit migrates more slowly than Rad53, which is still present in the complexes from *hir* mutant cells. An asterisk (*) indicates an unidentified polypeptide.

(D) A summary of Asf1-TAP mass spectrometry data. The percent sequence coverage of each protein is shown, with the number of unique peptides in parentheses.

These data also suggested that the HIR complex may not form in the absence of any subunit. To test this idea, the Hir2-STAP purification was performed from yeast strains lacking individual *HIR* genes (Figure S3B). In the absence of either Hir1 or Hir3, the remaining subunits still associated with Hir2. However, Hir3 was not detected by mass spectrometry in Hir2-STAP complexes from *hpc2Δ* cells, suggesting that Hir3 associates with Hir1 and Hir2 via an interaction with Hpc2 or becomes less stable in the absence of Hpc2. Therefore, although the HIR complex is not completely dissociated when individual components are missing, all Hir subunits are required for normal interaction with Asf1.

To assess the functionality of HIR complexes lacking individual subunits in vivo, interaction of Hir2 with the *HTA1-HTB1* promoter was investigated by using chromatin immunoprecipitation. The Hir proteins are negative regulators of the *HTA1-HTB1* locus [1, 2], repressing promoter activity outside of S phase [21, 22]. We observed that the Hir2-TAP protein was enriched 2.9-fold at the *HTA1-HTB1* promoter relative to a nonspecific locus, *ACT1* (Figure S3C), confirming that Hir2-STAP binds the promoter in vivo [22]. However, in the absence of any of the other three *HIR* genes, Hir2-TAP no longer specifically associated with the *HTA1-HTB1* promoter. In contrast, Hir2-TAP was enriched at the promoter 2.3-fold relative to *ACT1* in *asf1Δ* cells. We conclude that association of the HIR complex with the *HTA1-HTB1* promoter in vivo is dependent on Hir1, Hir3, and Hpc2, but not Asf1. In accordance with these data, previous studies have shown that transcriptional repression of *HTA1* by Hir2 is fully dependent on Hir1 and partially dependent on Hir3 [23]. However, because HIR complex binding to the promoter is independent of Asf1, the deregulation of histone gene expression in *asf1Δ* cells [9] is not due to the inability of the HIR complex to interact with promoter DNA. Instead, increased histone gene expression in *asf1Δ* cells may be due to altered chromatin structure at the promoter and/or may result from indirect defects on cell cycle progression [24].

Replication-Independent Chromatin Assembly by the HIR Complex and Asf1

Genetic and biochemical experiments had suggested that the yeast HIR complex would function as a histone deposition factor in conjunction with Asf1 [6, 7, 25]. To test this idea, we purified native HIR complexes by using gentle elution conditions via an EGTA-elutable TAP tag (CBP-TEV-ZZ, [15]; Supplemental Experimental Procedures). We used an electrophoretic mobility shift assay

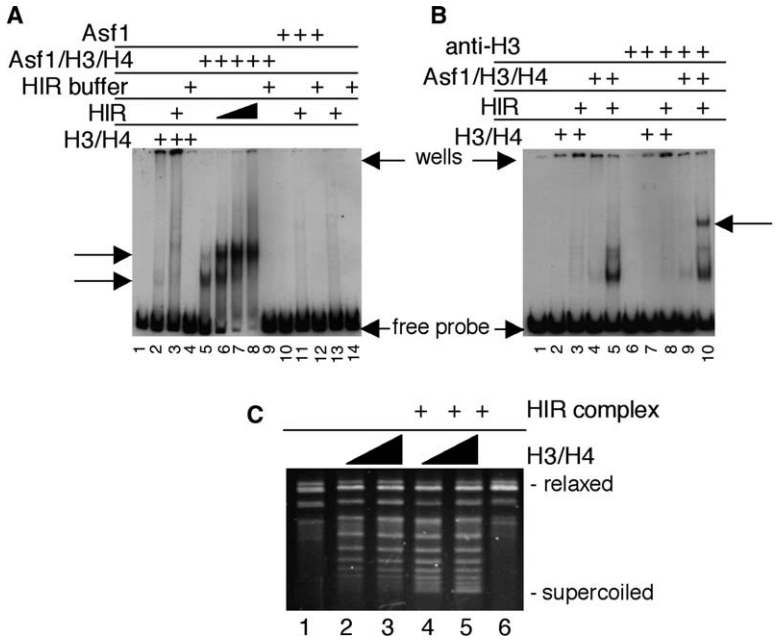

Figure 2. Histone Deposition by HIR and Asf1-H3/H4 Complexes

(A) 30 fmol of a 250 bp radiolabeled DNA probe was incubated with 100 fmol of histones H3/H4 (lanes 2–4), 100 fmol of preformed Asf1-H3/H4 complex (lanes 5–9), 100 fmol of Asf1 (lanes 10–12), and either 1.75 μl (lane 6), 3.5 μl (lane 7), or 7 μl (lanes 3, 8, 11, and 13) of HIR complex or HIR dialysis buffer (7 μL; lanes 4, 9, 12, and 14). Reaction products were resolved on a native 4% polyacrylamide gel and detected by autoradiography. The upper and lower arrows indicate two distinct shifted species.

(B) The shifted species contain histone H3. The EMSA was performed as described above, except that 5 μl of HIR complex or HIR dialysis buffer was used. Anti-histone H3 antibody (0.125 μg, Abcam) was added to lanes 6–10. The arrow indicates the supershifted species.

(C) Plasmid DNA prerelaxed with human topoisomerase I was incubated with CBP-TAP-purified HIR complex (20 μL, lanes 4, 5, and 6) preincubated with 0.4 (lanes 2 and 4) and 0.8 (lanes 3 and 5) pmol of histones H3/H4. DNA was analyzed by agarose gel electrophoresis and visualized by SYBR Gold (Molecular Probes) staining.

(EMSA) to detect formation of nucleoprotein complexes. When a radiolabeled 250 bp DNA probe was incubated with histones H3/H4 (Figure 2A, lane 2), a small amount of two slower-migrating species were observed in the gel (arrows), suggesting inefficient formation of histone-DNA complexes. The addition of the HIR complex slightly increased the amounts of these complexes (Figure 2A, lane 3), suggesting a mild stimulation of histone deposition. When Asf1-H3/H4 complexes were incubated with the DNA, more of the two shifted DNA species appeared (Figure 2A, lane 5), with the predominant shifted band being the faster migrating of the two species. Addition of increasing amounts of the HIR complex to Asf1-H3/H4 resulted in greater amounts of probe shifted (Figure 2A, lanes 6–8) and a higher proportion of the slower-migrating species. The observed shifts were dependent on histones H3/H4, because Asf1 or the HIR complex did not shift the DNA in the absence of histones (Figure 2A, lanes 10, 11, and 13). We therefore hypothesized that the two shifted species represent histone H3/H4-DNA complexes. Upon addition of anti-H3 antibodies to these reactions, a novel band of slower migration was formed specifically in reactions containing both the HIR complex and Asf1-H3/H4 (Figure 2B, lane 10). These data confirm that the HIR and Asf1-H3/H4 complexes together promote histone deposition.

To determine whether Asf1 was essential for histone deposition by the HIR complex, histones H3/H4 without Asf1 were incubated with plasmid DNA in the presence or absence of HIR complex (Figure 2C). To complete nucleosome formation, H2A/H2B were also added. Topoisomerase I was present to relax all unrestrained supercoils, so that any supercoiling of the DNA could be attributed to nucleosome formation. We observed that the HIR complex stimulated supercoiling under these conditions. These data indicate that the HIR complex alone is capable of replication-independent histone deposition.

We next determined whether histone deposition by Hir/Asf1 proteins would occur during DNA replication. Nucleosome formation was monitored by the extent of supercoiling of plasmid DNA replicated in a human cell extract. A radioactive deoxyribonucleotide was incorporated into replicated DNA molecules, distinguishing them from nonreplicated molecules. The addition of CAF-1 to this reaction results in preferential supercoiling of the radioactive, replicated molecules ([26–28]); Figure 3A, lanes 1–3), indicative of nucleosome formation on the replicated DNA templates. Hir protein complexes did not alter supercoiling of either the replicated or total DNA populations (Figure 3A, lanes 4 and 5). However, when preformed Asf1-H3/H4 complexes were added to reactions containing HIR complex, supercoiling increased almost 2-fold in the total DNA visualized by ethidium bromide (lanes 7 and 8). However, comparison of the replicated molecules (bottom) to the total DNA (top) showed that there was no preferential supercoiling of replicated molecules in the presence of the Asf1-H3/H4 and HIR complexes. No supercoiled DNA was produced in the presence of either an equivalent amount of Asf1-H3/H4 alone (Figure 3A, lane 6) or in the presence of the HIR complex with an equivalent amount of histones in the absence of Asf1 (Figure 3B, lane 3). These data confirm that Asf1-H3/H4 and the HIR complex together promote replication-independent histone deposition. Notably, recent experiments [29] demonstrate that both Asf1 and the HIR complex are important for replication-independent redeposition of histones at the yeast *PHO5* promoter, consistent with our biochemical observations.

Asf1-H3/H4 stimulates replication-linked histone deposition by CAF-1 ([7, 10, 11] and Figure 3B, lanes 5, 9, and 10). Because mutations in either the *HIR* or *ASF1* genes cause synthetic growth and silencing defects in the absence of CAF-1 subunits [7, 13], we hypothesized that the HIR complex might affect CAF-1 activity. This idea was tested in replication-coupled chromatin assembly assays containing a substoichiometric amount of CAF-1. However, the addition of HIR

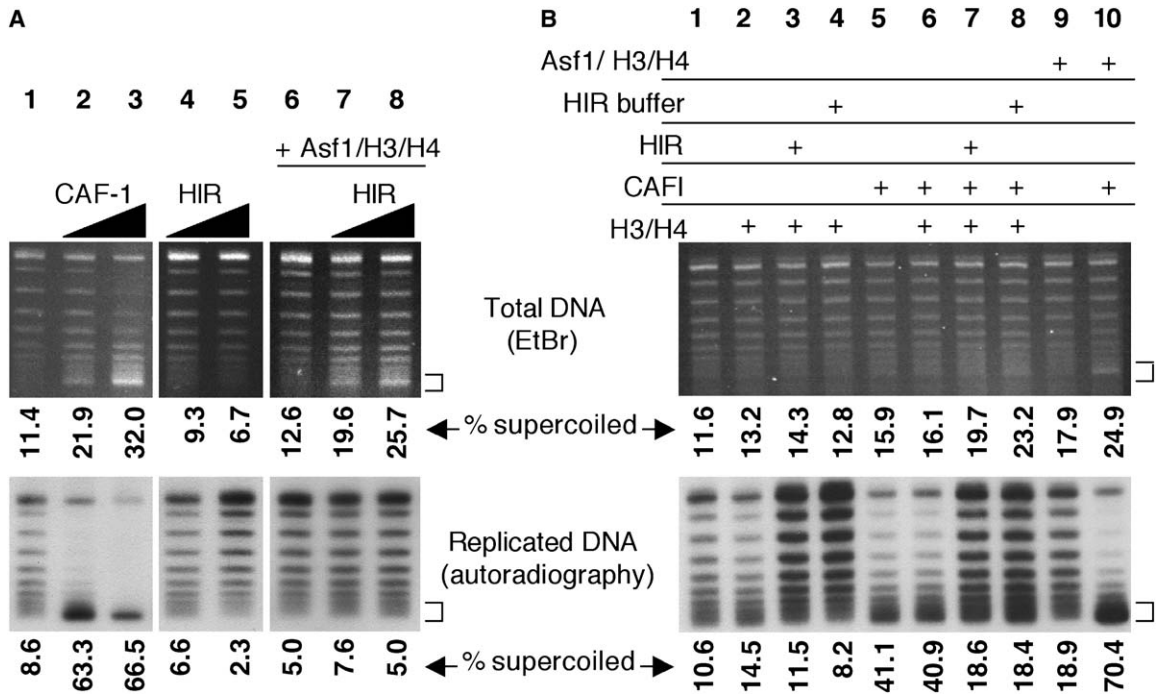

Figure 3. The HIR Complex and Asf1 Assemble Chromatin Independently of DNA Replication or CAF-1

(A) SV40 replication reactions containing α-^{32}P-dATP. CAF-1 (10 ng, lane 2, or 20 ng, lane 3) or CBP-TAP-purified HIR complex (2.25 μL, lanes 4 and 6, or 4.5 μL, lanes 5 and 7) was added as indicated. Asf1-H3/H4 complexes (0.9 pmol) were added in lanes 6–8. Chromatin assembly was detected by the appearance of supercoiled DNA (indicated by arrow) on an agarose gel. Total DNA was visualized by staining with ethidium bromide (top). Replicated molecules that incorporated α-^{32}P-dATP were detected by autoradiography (bottom). The values shown indicate the percentage of supercoiled DNA in each lane for both the total DNA and the replicated DNA.

(B) The HIR complex does not stimulate replication-dependent, CAF-1-mediated chromatin assembly. SV40 replication reactions were performed as in (A) in the presence of CAF-1 (7.5 ng, lanes 5-8, 10), histones H3/H4 (5 ng, lanes 2–4 and 6–8), HIR complex (4 μL, lanes 3 and 7), HIR dialysis buffer (4 μL, lanes 4 and 8), and preformed Asf1-H3/H4 complex (3.6 pmol, lanes 9 and 10).

complex preincubated with histones H3 and H4 did not significantly increase the amount of supercoiled DNA (Figure 3B, compare lanes 6, 7, and 8). Although the percentage of supercoiled replicated DNA appeared to decrease with the addition of the HIR complex to CAF-1, this was likely due to stimulation of DNA replication in the presence HIR buffer components (lanes 7 and 8). In contrast, this same amount of HIR complex triggered replication-independent histone deposition in the presence of Asf1-H3/H4 (Figure 3A). Therefore, the HIR/Asf1 proteins are sufficient for replication-independent chromatin assembly and operate independently of CAF-1. Furthermore, these data provide a biochemical distinction between the activities of the HIR complex and Asf1

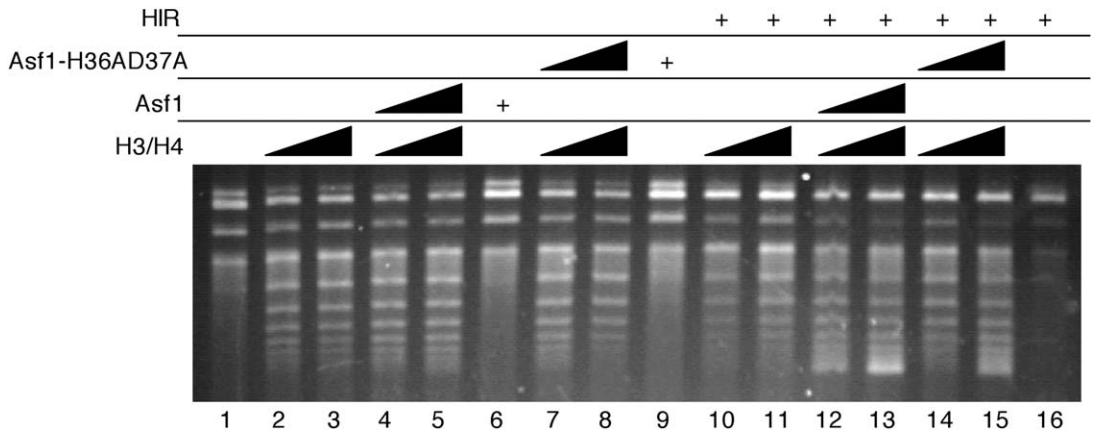

Figure 4. Asf1-H36AD37A Impairs Histone Deposition by the HIR-Asf1 Complex

Histone deposition was assayed as in Figure 2C. Plasmid DNA prerelaxed with human topoisomerase I was incubated with 0.4 and 0.8 pmol histones H3 and H4 (lanes 2 and 3), 0.8 and 1.6 pmol Asf1 (lanes 4 and 5), or Asf1-H36AD37A (lanes 7 and 8) preincubated with 0.4 and 0.8 pmol histones H3/H4. CBP-TAP-purified HIR complex (15 μL) was added to reactions in lanes 10–16.

despite their similar genetic phenotypes and physical association.

HIR Binding Mutations in Asf1 Inhibit Histone Deposition

Physical interaction between human Asf1a and the Hir protein homolog HIRA is disrupted by mutation of a small region of conserved residues on the surface of Asf1a [30]. Mutation of the homologous residues in budding yeast blocks the ability of Asf1 to contribute to telomeric gene silencing, suggesting that direct interaction between Asf1 and the HIR complex is important for silencing [31]. We therefore predicted that histone deposition by Asf1-HIR complexes would be impaired by the H36A,D37A mutations that affect the Asf1-HIR interaction. To test this idea, we purified recombinant Asf1-H36AD37A and tested its replication-independent chromatin assembly activity. As observed previously (Figures 2 and 3), wild-type (wt) Asf1 promoted DNA supercoiling by the HIR complex (Figure 4, lanes 12 and 13), indicating increased histone deposition. In contrast, the Asf1-H36AD37A mutant stimulated histone deposition by the HIR complex (lanes 14 and 15) less efficiently. However, Asf1-H36AD37A promoted histone deposition in the absence of the HIR complex at a level comparable to wt Asf1 (Figure S4). Therefore, the Asf1-H36AD37A mutant was not intrinsically defective for histone deposition but could not be stimulated by HIR complexes as well as wt Asf1. These data indicate that a direct physical interaction between Asf1 and the HIR complex is important for their combined histone deposition activity.

Conclusions

Hir1, Hir2, Hir3, and Hpc2 together comprise the HIR complex, a novel macromolecular chromatin assembly factor that copurifies with the chromatin assembly factor Asf1. Asf1 and the HIR complex together promote replication-independent histone deposition in a manner stimulated by their interaction. The Asf1/Hir proteins thus form a conserved histone deposition pathway [6, 7, 25, 32] implicated in histone replacement during RNA polymerase movement [33] and reassembly of promoters after histone eviction [29]. Because all components of the yeast HIR complex have been identified here, this study provides the groundwork for mechanistic analysis of replication-independent chromatin assembly in a genetically tractable organism.

Studies from the Workman laboratory describe an additional function of the HIR complex, formation of a SNF/SWI-resistant nucleoprotein structure, consistent with the role of Hir proteins in histone gene repression [34]. We note that our preparations of the HIR complex display the same polypeptide composition.

Experimental Procedures

All methods are described in the Supplemental Experimental Procedures.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and two figures and are available with this article online at http://www.current-biology.com/cgi/content/full/15/21/2044/DC1/.

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